

IDENTIFYING MECHANISMS REGULATING WNT SIGNALING DURING  
POSTEMBRYONIC DEVELOPMENT

Kelly Marie Alexandre

A thesis submitted to the faculty of the University of North Carolina at Chapel Hill  
in partial fulfillment of the requirements for the degree of Master of Science in the  
Department of Biology

Chapel Hill  
2009

Approved by:

Mark Peifer

Victoria Bautch

Greg Copenhaver

## ABSTRACT

Kelly Alexandre: Identifying Mechanisms Regulating Wnt Signaling During  
Postembryonic Development  
(Under the direction of Mark Peifer)

Proper regulation of the Wingless (Wg)/Wnt pathway is essential for development; inappropriate activation of the pathway occurs in many human cancers. Wg signaling stabilizes the effector, Armadillo (Arm) and in its absence Arm is phosphorylated, ubiquitinated, and then degraded. In this work, we take a closer look at Arm regulation. First we investigated the difference in Arm accumulation in APC, a member of the destruction complex, mutants. We find that while protein levels of Arm are the same, mRNA levels differ between embryos and larvae. Second, published data suggests that the SCF complex is responsible for Arm ubiquitination. The canonical SCF complex is known to contain five parts, including a RING domain protein of the Roc1/Rbx1 family. However, some data has called into question the exactly which Roc protein is part of the complex. Our data suggests that neither Roc1b nor Roc2 are solely responsible for Arm degradation.

To Shigeru Miyamoto and Chris Alexandre, two artists who changed my life

## ACKNOWLEDGEMENTS

I would like to begin by thanking my advisor, Mark Peifer. His guidance and support have helped me grow as a scientist and a person. I would also like to thank the Peifer lab—Dave, Nasser, Ed, Jess, Nathan, Steph, and Doug. We have rejoiced over scientific accomplishments and commiserated over the failed experiments. Jess and Steph in particular have become a large part of my life in and outside of lab. My committee has been indispensable in my progress as a scientist. Of the people in my life there are a few who I could not be without, whose love and support have shaped who I am today. My parents have played integral parts in shaping my life and helping me along in my journey. I am lucky enough to have four loving, supportive, and amazing parents—Wayne, Debbie, Tom and Maria. They have had four different lives and their diversity has created a wealth of knowledge that I continually tap into. While I was growing up their support and understanding was there for me through some very hard times. Now, as an “adult” I no longer need that constant guidance but I will always appreciate their love. Next, I want to acknowledge Matt. He has been my personal cheerleader for the past three years. Each time I thought things would never get better he was there to show me the light. His love helps me to understand I am truly blessed. And of course, I have to thank my brother, Chris. The two of us make up a twenty-eight year, two person mutual admiration society. He has always been my biggest fan and supporter. In turn I am in

constant awe of his artistic ability and the fierceness of his love for his family. I think my mom said it best, "It's a good thing I had two of you. Otherwise nobody would understand you." And now it is the beginning of a fantastic story!

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## LIST OF ABBREVIATIONS

aa	amino acid
APC	Adenomatous Polyposis Coli
Arm	Armadillo
ATP	Adenosine triphosphate
$\beta$ -cat	$\beta$ -catenin
bp	base pairs
Ci	Cubitus interruptus
CK1	casein kinase 1
Cul1	Cullin1
DNA	deoxyribonucleic acid
Dvl	disheveled
FAP	familial adenomatous polyposis
FLP	Flippase recombination enzyme
FRT	Flippase Recognition Target
GFP	green fluorescent protein
kb	kilobase
kd	kilodalton
MCR	mutation cluster region
mRNA	messenger ribonucleic acid
mt	microtubule
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
SCF	Skip Cullin F-box



RT-PCR	reverse transcriptase polymerase chain reaction
Skp	Skip
Slmb	Slimb
TBST	Tris-Buffered Saline Tween-20
TCF	T-cell factor
Wg	Wingless
Wnt	wingless/INT
wt	wild type
yw	yellow white

## Introduction

The process of normal development is one of the biggest and most exciting questions in science. Understanding how a single cell can give rise to a functional, sexually mature adult has long been an important task of scientists. As more has been learned about development, several unifying commonalities have been discovered. One of these is that developmental progression depends on a cell's ability to produce and respond to various signals. Certain critical signaling pathways are evolutionarily conserved across multiple species. Studying and understanding how these pathways function in different animals gives us a broader insight into development as a whole.

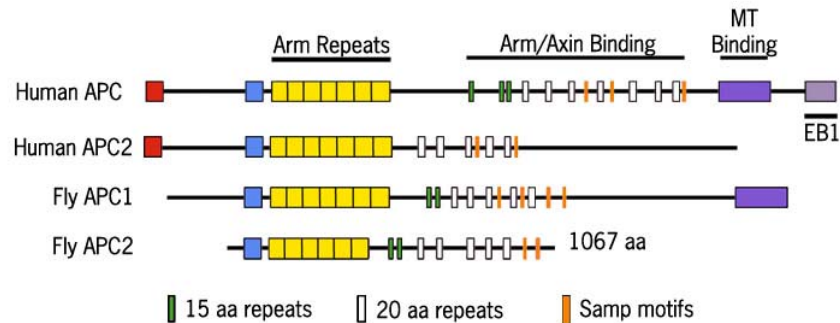
In particular, the Wnt (wingless/INT) pathway is highly conserved and is vital for proper development. Wnt ligands were originally discovered in mice as oncogenes, and simultaneously their roles in development were elucidated in *Drosophila melanogaster* embryos and adults (Nusse and Varmus, 1982; Nusslein-Volhard and Wieschaus, 1980; Sharma and Chopra, 1976). The ventral epidermis of the developing fruit fly embryo was a particularly good model system for studying this event. Each segment has an alternating pattern of posterior cells that secrete naked cuticle and anterior cells that make small hairs called denticles. Wingless (Wg), the fly orthologue of Wnt1, is the segment polarity gene that helps to create this pattern (Nusslein-Volhard and Wieschaus 1980; Rijsewijk, 1987). One row of cells per segment secretes Wg, and thus sets

up a gradient (van de Wetering, 1997). The cells that receive Wg produce naked cuticle. Cells that don't receive Wg produce denticles. When the gradient is missing, is not set up properly, or the receiving cells are unable to respond to the signal, all cells produce denticles (Perrimon and Mahowald 1987). In cases where Wg or its signal transduction pathway are inappropriately activated, all cells secrete naked cuticle. Both circumstances result in embryonic lethality. Wnt signaling has been shown to be important in other animals, as well. For example, *Xenopus* embryos develop a second body axis when early embryos are injected with active Wnts (McMahon and Moon, 1989).

Subsequently the Wnt pathway has been explored in humans. Inappropriate activation of key developmental signaling pathways, such as the Wnt, Hedgehog or receptor tyrosine kinase pathways, drive most cancers. Activation of the Wnt pathway is present in many cancers (Polakis 2007). For example, more than ninety percent of colon cancers have a mutation in the Wnt pathway. Typically, the cancerous cells have lost regulation of Wnt signaling so that it is constitutively turned on.

According to the American Cancer Society, colorectal cancers are the third most common type of cancer in the United States (2008). There are two types of colorectal cancer, inherited and sporadic. The inherited type is responsible for 15% of these cancers; one inherited syndrome is called familial adenomatous polyposis (FAP). Inherited *Adenomatous polyposis coli* (APC) germline mutations have been shown to be essential for FAP. Studies have also shown that truncations and other mutations in APC proteins are also responsible

for the majority of sporadic colorectal cancers, which comprise the remaining 85% (Polakis, 2007).

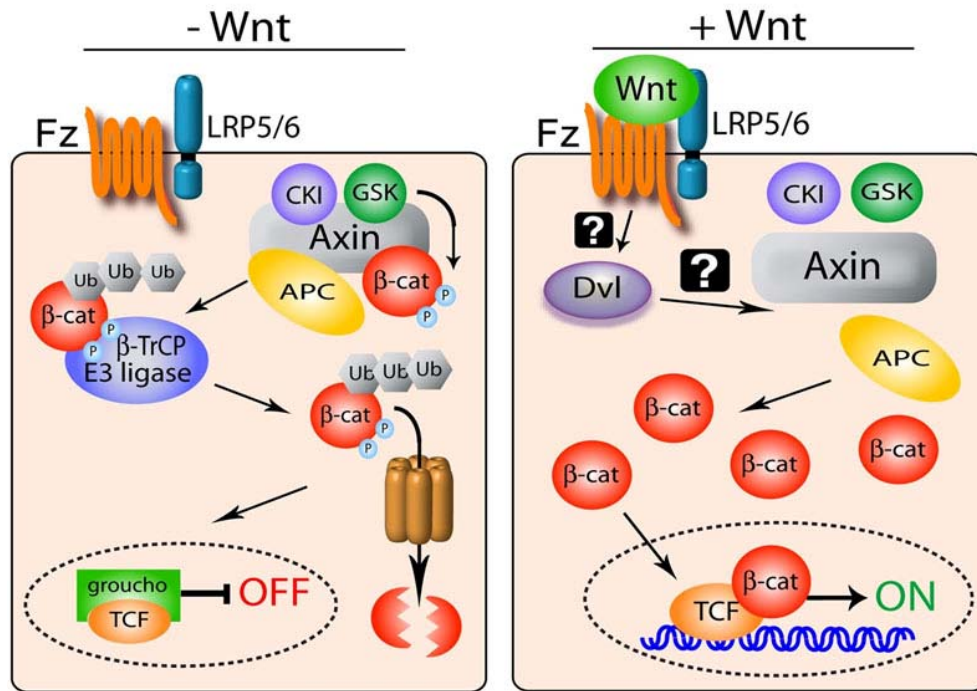


### Figure 1. APC comparison

There are two APCs in humans and in flies. APCs from both species contain Armadillo repeats for protein protein interactions, 15 and 20 amino acid repeats that can bind Arm/ $\beta$ -cat, and SAMP motifs for binding Axin. Human APC and fly APC1 also contain a microtubule binding domain. Human APCs have an oligomerization domain that allows them to bind one another; however, the fly APCs have not been shown to possess such a domain.

APC was originally characterized in 1991. By analyzing DNA from 40 patients that had FAP, scientists were able to map the *APC* gene to chromosome 5q 21-22; from there they narrowed the region down to what is now known as the *APC* gene (Grodin et al., 1991). APC was later shown to be a key component of the destruction complex that forms to target  $\beta$ -catenin ( $\beta$ -cat), the ortholog of fly Armadillo (Arm), for destruction (Rubinfeld et al., 1995). Inactivation of APC stabilizes  $\beta$ -catenin and activates Wnt signaling. In many animals, there are two APC family members: APC1 and APC2 (Fig 1). APC proteins are a mosaic of protein binding sites: Armadillo repeats for protein-protein interactions with diverse partners, 15 and 20 amino acid repeats that have the ability to bind Arm/ $\beta$ -cat, and SAMP motifs that bind Axin, another component of the destruction

complex. Mammalian APC also contains an oligomerization domain and a microtubule binding region. In colon tumors, one allele of *APC* is usually inactivated while the other produces a truncated protein (Polakis, 2007). Most of



**Figure 2 The Wnt Pathway**

The first diagram depicts a cell not receiving Wnt signal. Arm is phosphorylated, ubiquitinated, then degraded. The second half is a cell that has received Wnt. Dvl inactivated the destruction complex, Arm accumulates, moves into the nucleus, and turns on Wnt responsive genes. (adapted from Roberts et al 2007)

the truncations found to be critical in cancer occur in a portion of the gene that has been dubbed the Mutational Cluster Region (MCR). This region encompasses two of the twenty amino acid repeats, and conserved region B, and ends just before the SAMP motifs. Other roles for APC beyond canonical Wnt signaling have also been proposed, such as roles in chromosome stability, cell adhesion, and regulation of actin and microtubules (Nathke, 2006).

The precise details of how the Wnt pathway functions are still being examined. However, the basic steps are known (Cadigan, 2008). The key regulated effector is Arm. It is typically found both in the cytoplasm and at cell-cell junctions, where it serves a different role in cell adhesion. When a cell receives Wnt signal, Arm is able to relocate into the nucleus. Until this point, the HMGbox DNA binding proteins of the T-cell factor (TCF) family and co-repressor Groucho are actively repressing transcription of Wnt responsive genes (Cavallo et al., 1998). Once inside the nucleus, Arm can displace Groucho, bind with TCF and recruit transcriptional co-activators (Brunner et al., 1997; van de Wetering et al., 1997).

In a cell that is not receiving Wnt, the destruction complex is formed (Cadigan, 2008). This complex is comprised of APC, Axin, Casein kinase 1 (CK1), and GSK-3. Arm binds Axin and APC, and CK1 and GSK-3 phosphorylate Arm in a series of phosphorylation events, each priming the site for the subsequent phosphorylation. During this period, APC is also phosphorylated. Once both proteins are phosphorylated, Arm is shuttled to an E3 ubiquitin ligase and ubiquitinated (Aberle et al., 1997). This ubiquitination allows the 26S proteasome to recognize Arm and then degrade it, thus maintaining repression of Wnt responsive genes.

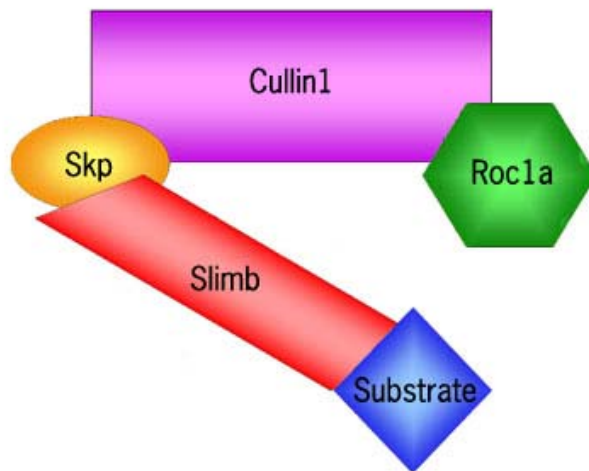
In a cell that receives Wnt signal, the cell surface receptor LRP5/6 (Arrow), a single-pass transmembrane protein, and Frizzled, a seven-pass transmembrane protein, form a complex and bind Wnt (Cadigan, 2008). In parallel, Disheveled (Dsh) is activated, can interact with Frizzled, and disables

the destruction complex; the mechanism by which this occurs is not yet fully understood. CK1 also phosphorylates Arrow, which then recruits Axin to the receptor complex. This recruitment helps to disable the destruction complex. With no destruction complex to begin the degradation of Arm, Arm accumulates in the cytosol. From there it is able to move into the nucleus, remove the repressor Groucho, and turn on Wnt responsive genes.

The Wnt pathway is one of many pathways regulated by ubiquitin-mediated proteolysis. Ubiquitin ligases have many functions in cells (for a review see Petroski, 2008). For example, they have been shown to regulate hypoxia responsive transcription factors, mitosis, the cytoskeleton, cell cycle transitions, and protein degradation. The general method of protein degradation begins when an E1 protein uses ATP to activate a ubiquitin moiety. The ubiquitin is then transferred from the E1 to an E2 protein. Finally, the E3 facilitates the transfer of the ubiquitin from the E2 to the substrate. This last step can be accomplished in different ways. In HECT domain-containing E3's, the ubiquitin is transferred directly to the E3 and then to the substrate. In RING domain-containing E3's, the transfer is only stimulated by the E3 and the ubiquitin is passed from the E2 to the substrate.

Arm is thought to be degraded by the Skip Cullin F-box (SCF) complex (for a review see Willems, 2004). The canonical SCF complex is comprised of Cullin 1 (Cul1) that can bind Roc1 on its carboxyl-terminus and Skip 1 (Skp) on the amino-terminus (for a review see Ho, 2006). Roc1 is a RING domain protein that provides specificity for the E2 protein. Skp binds a member of the large F-

box protein family; in the regulation of  $\beta$ -cat, that F-box protein is Slimb (Slmb). Slmb binds Skp on its amino-terminus and a substrate such as Arm on the carboxyl-terminus. The phosphorylation of Arm by the destruction complex is essential for Arm to be recognized by the SCF complex.  $\beta$ -TrCP, the human ortholog of Slmb, can pull down  $\beta$ -cat and APC in a co-immunoprecipitation assay (Hart et al., 1999).



**Figure 3 The SCF complex**

Consistent with this model, when Slmb or Cul1 mutant clones are made in fly imaginal discs (Jiang and Struhl 1998; Ou, 2002), Arm accumulates in the cytosol, suggesting that it is not being degraded. This implies that the SCF complex is responsible for targeted degradation of Arm. However, work from the Duronio lab showed that when similar loss of function mutant clones are made with Roc1a, Arm does not accumulate (Noureddine et al., 2002). As Roc1a is part of the canonical SCF complex, and other components in the complex have already been shown to play a role in the degradation of Arm, these data raise



questions about the mechanism of Arm degradation and the role the canonical SCF complex plays in it.

There are two classes of Roc proteins: Roc1 and Roc2, with most metazoans having one of each (for a review see Yi Sun, 2001). *D. melanogaster* has three Roc proteins: Roc1a, Roc1b and Roc2 (Donaldson et al., 2004, Nouredine, 2002). Roc1a and hRoc1 have 100% identity in the RING domain. These data have led us to consider two hypotheses to explain the aforementioned discrepancy. First, it is feasible that the Roc proteins have redundant functions in *D. melanogaster*. Alternatively, it may be that a different Roc protein than Roc1a functions in the fly SCF complex to degrade Arm. We investigated both of these possibilities by analyzing the accumulation of Arm in single Roc mutants.

Our lab has long been interested in the Wnt pathway. While analyzing *Drosophila* APC mutants, we perceived interesting differences in Arm accumulation in different tissues. In fly embryos that lack both APC1 and APC2, Arm is not targeted for destruction and accumulates at very high levels as compared to wild type (Akong et al., 2002). In contrast, while *Apc1 Apc2* mutant cells in larval wing discs or brains have an elevated accumulation of Arm, this accumulation is much less dramatic than what was seen in the embryos (unpublished data and Hayden, 2007). This was puzzling as it has been reported that mutations in larval imaginal discs of the destruction complex, such as Axin or Slmb, result in very high level accumulation of Arm, as was seen in the embryo (Hamada et al., 1999; Jiang and Struhl, 1998). This led us to explore the

difference between the embryo and wing discs phenotypes, testing the possibility that APC might not be fully required for destruction complex function in all tissues.

## Results

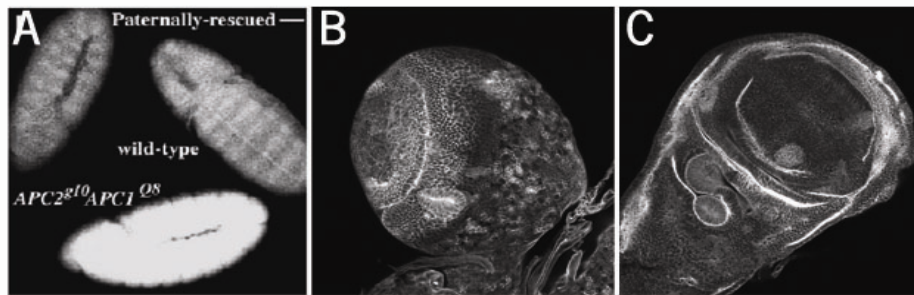
### Armadillo regulation through development

The Wnt signaling pathway has been studied extensively. This wealth of knowledge has allowed us to compare not only data generated in our lab but also other published results. Our lab noticed that *APC2<sup>G10</sup>APC1<sup>Q8</sup>* mutant clones, *APC2* and *APC1* null alleles, generated in imaginal discs (Fig 4C) or in the optic lobes of the brain (Fig 4B, Hayden et al, 2007) did not appear to accumulate Arm at levels as high as those reported by other labs who had examined clones mutant for Axin or Slmb (Hamada et al, 1999; Hart et al, 1999; Jiang and Struhl, 1998). In addition, when we compared the levels of Arm accumulation between *APC2<sup>G10</sup>APC1<sup>Q8</sup>* embryos and *APC2<sup>G10</sup>APC1<sup>Q8</sup>* mutant wing disc or brain clones the levels of Arm accumulation in the wing disc clones (figure 4B, C) were much lower than those of the embryos (Fig 4A, McCartney et al 2006, Akong et al 2002). These data suggested the possibility that there could be another protein acting in the same capacity as APC, helping to regulate Arm levels.

Alternatively, Arm might be regulated differently in embryos than in wing discs.

To distinguish between the possibilities, Daniel Schneider and David Roberts started by making wing disc clones mutant for other destruction complex proteins, including Axin and Slmb. Surprisingly, immunostaining of the wing discs revealed that the levels of Arm in the different clones were equivalent to the lower levels we observed when mutant for both APCs (figure 5). As Arm is also

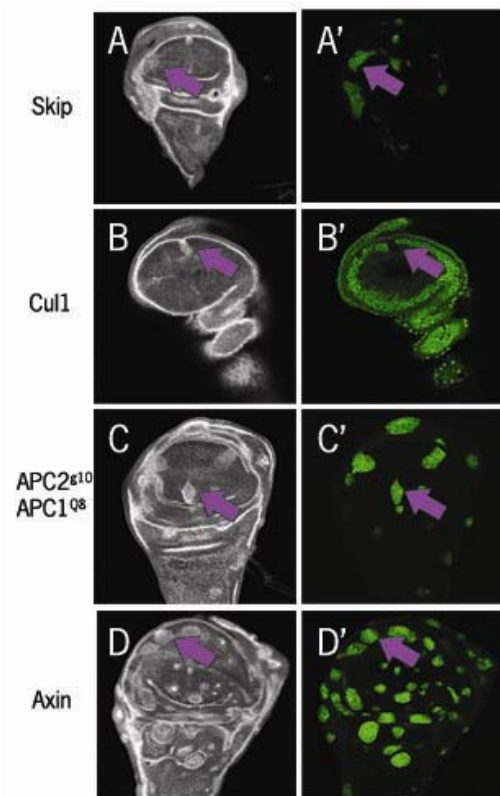
localized to the cellular junctions, we now suspect the apparent high level of accumulation others previously reported was due to the plane of focus. Loss of the destruction complex alters the morphology of wing disc cells, and an image taken at the apical portion of the cell will show a higher level of Arm than a more



**Figure 4 Armadillo accumulation in  $APC2^{g10}APC1^{Q8}$  mutants during fly development**  
A. (McCartney et al., 2006) Paternally rescued and wild type embryos (stage 9) show similar levels of Arm accumulation; whereas the double mutant embryo has a much higher than normal accumulation. B. mutant clones in the 3<sup>rd</sup> instar larval brain & C. mutant clones in the 3<sup>rd</sup> instar larval wing disc; these both display a modest accumulation of arm above normal levels. B. & C. taken by Kuo-Chen Jung

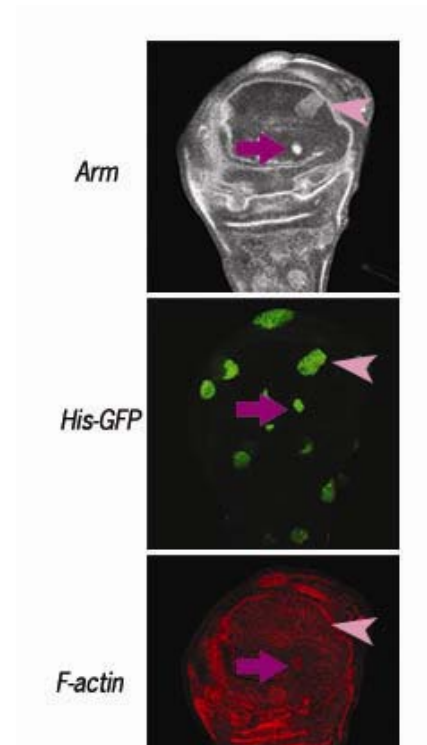
basal section (figure 6); the same is seen in wildtype cells. This still leaves the question about why we observed such a striking difference in Arm accumulation between embryos and larval stages. I addressed this issue. Before I could conduct further analysis of the mutant phenotypes, it was necessary for me to characterize Arm levels through development. I began by comparing Arm protein levels of wild type stage 9 embryos, stage 17 embryos, and wing discs and brains of third instar larvae (Fig 7A). Arm is known to be upregulated in stage 9 embryos as segment identities are defined. By stage 17, Arm is downregulated in the epidermis as the embryo prepares to hatch (Peifer, 1993; Riggelman B et al., 1989). As anticipated, the levels of Arm protein in the stage 17 embryo were

lower than in stage 9. When the stage 9 and the larval tissues were compared however, the amount of Arm was found to be roughly similar (Fig 7A). This result surprised us, because the levels of Arm accumulation in  $APC2^{G10}APC1^{Q8}$  mutants in the two stages are so different.



**Figure 5 Wnt pathway component clones**

A.-D. 3<sup>rd</sup> instar larval wing discs immunostained for Armadillo A., B., D. wing discs with GFP to mark the presence of mutant clones. C. the lack of GFP denotes the presence of a mutant clone. Arrows mark mutant clones. The rows denote the mutation in the clone cells. A., C., and D were taken by D. Schneider and D. Roberts



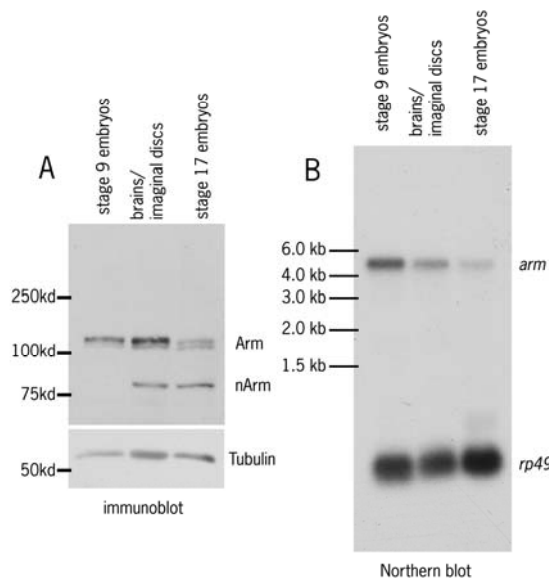
**Figure 6 Plane of focus**

Arrows mark the location of a clone taken at the apical part of the cell, note that the levels of all three channels are elevated. Arm is known to be enriched at apical junctions. Arrowheads mark a clone at a more basal section and levels of arm are elevated but at a much lower amount. Taken by Kuo-Chen Jung

In order to gain a deeper insight into this process, I next looked at mRNA levels of arm. I made mRNA preparations from wildtype animals from the three stages. I prepared a radioactive probe to arm and probed a Northern blot of all three stages to visualize the levels of mRNA, using the ribosomal protein gene

rp49 as a loading control (Fig 7B). A STORM machine was used to quantify the amount of mRNA in the three stages by measuring the amount of fluorescence. Levels of arm were normalized to the level of rp49. As expected, the stage 17 mRNA levels of arm were low.

However, when stage 9 and the larval tissues were compared, we found that arm mRNA levels in the embryo were about two times those in the brain and imaginal discs. The difference in mRNA levels suggests that there may be a different requirement for Arm for the embryos than in the larval tissues. It is possible that the rapidity of development in these two different stages changes the amount of *arm* mRNA that is needed to assure against potential problems.

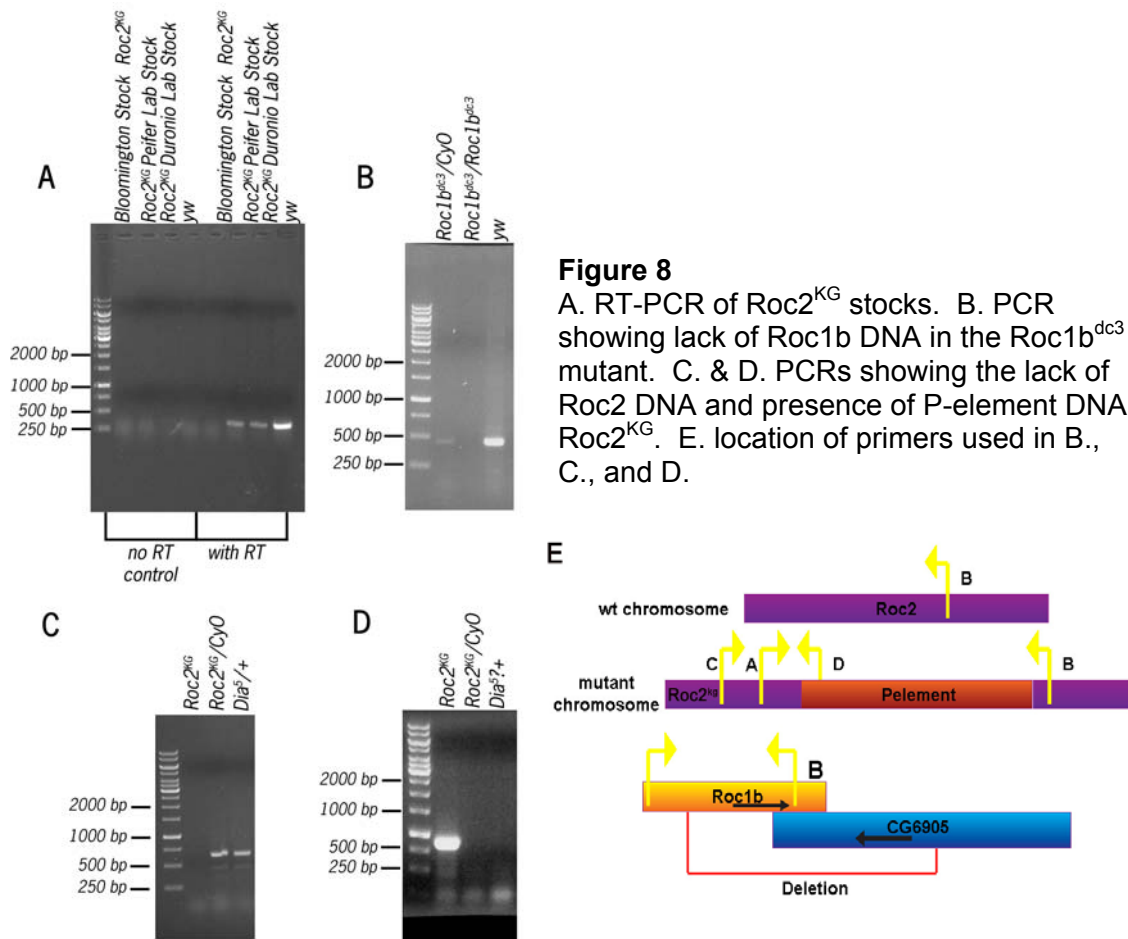


**Figure 7 Armadillo levels at different stages of development**  
A. Immunoblot comparing the amount of Armadillo protein levels. Stage 9 embryos and larval tissues have similar levels of protein.  
B. Northern blot comparing the amount of Armadillo transcript levels. Stage 9 embryos have almost twice as much *arm* mRNA as the larval tissues.

### Assessing the roles of different Roc proteins in Arm regulation

Degradation through ubiquitination is a common method of protein regulation in cells (for a review see Petroski, 2008). E3 ubiquitin ligases form

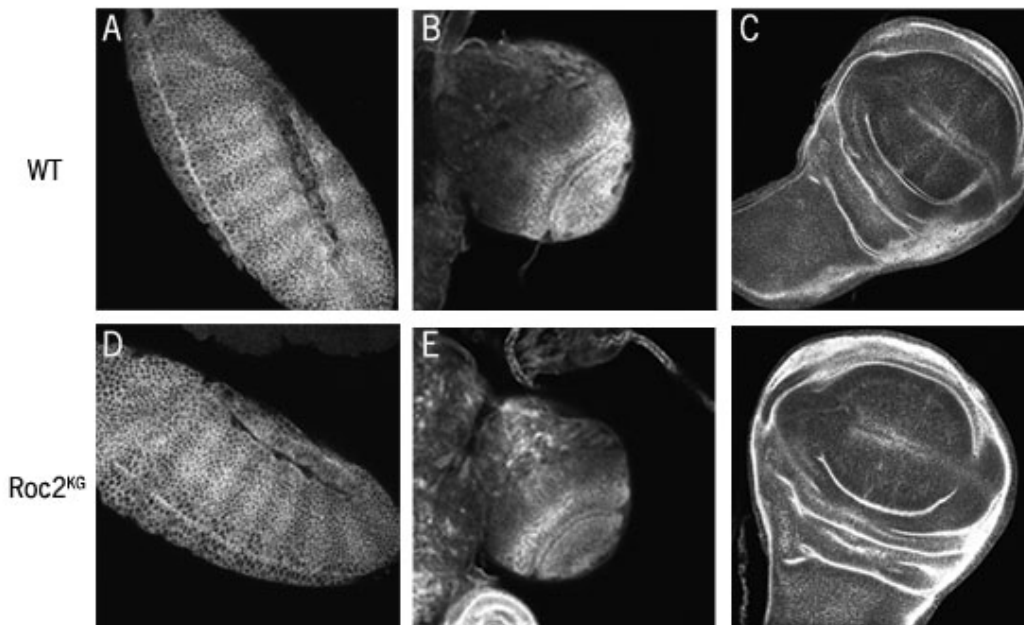
discrete complexes to target specific proteins. The composition of these complexes gives them their specificity. The canonical SCF complex consists of a Cul1 scaffold, Skp1 as an accessory protein to bind the F-box protein, an F-box protein that provides specificity for the substrate (in this case Arm), and Roc1a to provide specificity for the E2 protein (for a review see Ho et al., 2006). This SCF complex, with Slmb as the F-box protein, has been implicated in the targeted



degradation of Arm. Clones mutants for Cul1 or Slmb in the larval wing disc show an accumulation of Arm (Jiang and Struhl, 1998; Ou et al., 2002). In a pull-down assay the orthologue of Slmb,  $\beta$ -TrCP, comes down with APC and Arm (Hart et al., 1999). These data seem to implicate the SCF complex in targeted

Arm degradation. This possibility came into question, however, when it was shown that *Roc1a* mutant clones in larval wing discs do not show an accumulation of Arm above wild type levels, but do show accumulation of a different SCF target, the Hedgehog effector Cubitus Interruptus (Noureddine et al., 2002). Given these data, we wanted to find out if there was a different Roc protein in *D. melanogaster* that acts in the SCF complex, or if the three Rocs were functioning redundantly in this process.

To ascertain the mechanism of Arm destruction, we planned to test these hypotheses by analyzing Arm accumulation in mutants in each of these Roc proteins. The first mutant we looked at was *Roc2<sup>KG</sup>*, a null allele of *Roc2*, which was generated by a P-element insertion (Reynolds et al., 2008). The mutant is

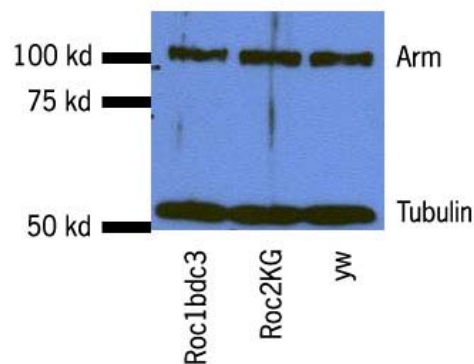


**Figure 9 Armadillo levels in *Roc2* mutants**

A.-C. are wildtype embryo and larval tissues D.-F. are homozygous mutant *Roc2<sup>KG</sup>* embryos and larval tissues. A. & D. are stage 9 embryos. B. & E., C. & F. are 3<sup>rd</sup> instar larval brains and wing discs, respectively

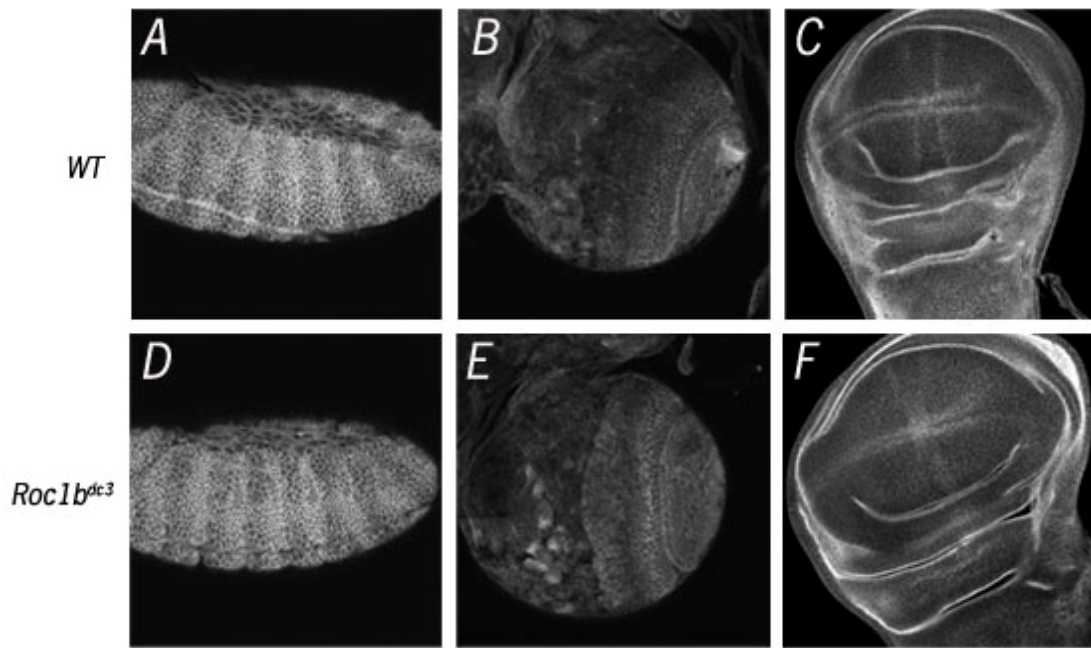


homozygous viable and fertile, suggesting that it is not an essential gene. Given the essential role of Wnt signaling and its regulation, this by itself would support the idea that the Roc proteins are acting redundantly, or that Roc2 is not part of the relevant SCF complex. This mutant does not produce a transcript of the gene (Reynolds et al., 2008), and we verified this by PCR and RT-PCR (Fig 8A, C, D). There had been some question about the phenotype of the stock we had in lab, because some of the phenotypes had changed so I tested several stocks to assess which had the correct mutation. The stock from the Bloomington Stock Center proved to be correct. We next immunostained three tissues from *Roc2<sup>KG</sup>* mutants to look at Arm accumulation. When compared to wild type, the mutant embryos, imaginal discs, and larval brains did not show any Arm accumulation beyond normal levels (Fig 9). A Western blot also indicated these animals also had the same amount of Arm protein as wildtype (Fig 10). Together, these data suggest that the SCF complex does not degrade Arm solely through the action of Roc2.



**Figure 10**  
Immunoblot comparison of  
Armadillo levels in Roc mutants

Next, we analyzed *Roc1b*. The mutant, *Roc1b<sup>dc3</sup>*, is a homologous recombination deletion that is homozygous viable but male sterile (Donaldson *et al* 2004). As with the *Roc2* mutant, we verified the mutation by PCR (Fig 8B). It did not show evidence of *Roc1b*, supporting the fact that this indeed a *Roc1b* mutant. Once verified, we immunostained embryos, wing discs, and larval brains to look for Arm accumulation. When compared to the wild type, *Roc1b<sup>dc3</sup>* mutants did not show accumulation above normal (Fig 11). A Western blot confirmed these results (Fig 10). Combined, these data point to *Roc1b* not being necessary for Arm degradation by itself.



**Figure 11 Armadillo levels in *Roc1b* mutants**

A.-C. are wildtype embryo and larval tissues D.-F. are homozygous mutant *Roc1b<sup>dc3</sup>* embryos and larval tissues. A. & D. are stage 9 embryos. B. & D., C. & F. are 3<sup>rd</sup> instar larval brains and wing discs, respectively

## Discussion

Our lab is fascinated by the process of development and the Wnt signaling pathway. We were intrigued when we compared published data and our own; and thought about the questions it brought up. Two of these questions are the ones I strove to answer with my experiments. The first was why was there a difference in the levels of Arm in the wing discs and brains of 3<sup>rd</sup> instar larvae, versus in embryos, when APC1 and APC2 are inactivated? The second question was raised by data generated by the Duronio lab; is the canonical SCF complex the primary mechanism for destroying Arm in *Drosophila*? These questions are both complex and we cannot hope to fully answer them in one paper. However, the experiments I have completed give us insights and ideas for future work that will one day explain part of the mystery of the Wnt pathway.

As mentioned before, we found a discrepancy between the levels of Arm accumulation during larval stages, when comparing our own data on APC double mutants and published data on mutants of other parts of the destruction complex and the Wnt pathway. We began to address this by recapitulating these clones in our lab. This revealed that all of these mutants all lead to an elevation of Armadillo accumulation. In contrast to the previously published data however, our clones display a much lower accumulation of Armadillo. Our data suggests this discrepancy may be due, in part, to the plan of focus when capturing the image. Since Arm can also be found at apical junctions, it is naturally enriched at

the apical portion of the cell. An image taken at this point appears “artificially” brighter than a more basal section of a neighboring cell. We believe changes in imaginal disc morphology caused by loss of destruction complex proteins can lead to this artifact.

It was unexpected that the stage 9 embryos and wing discs/brains would have the same levels of Arm protein but different levels of *arm* RNA. During embryogenesis, there are many vital processes and changes being set up in the fly. It could be that the embryo keeps extra transcripts waiting to accommodate the need for very rapid changes in signaling and to accommodate responses in case something inside the cell goes wrong. The embryos could then quickly make more Arm protein, without having to wait for additional transcription. This additional supply might not be necessary in the wing disc because the changes aren't occurring as rapidly. Our data suggests it is also a possibility that Arm is being degraded at a higher rate in the embryo than it is in the larva. There are points in development that require a large amount of Armadillo. At these points, the cessation of degradation would allow Armadillo to accumulate at a much higher rate than if the levels of transcript were lower.

Before undertaking these experiments we postulated that there may be another protein helping to regulate Arm in the wing discs. None of our data preclude that from still being a possibility. For example, in embryos there could be a protein that aids the destruction complex work faster or more efficiently. Further experiments will have to be done to look into this further

Based on our data thus far, it seems that neither *Roc1b* nor *Roc2* are solely responsible for Arm degradation in flies. Since Arm degradation is an essential process during development and both *Roc1b* and *Roc2* are viable, these data make sense. This does not rule out the possibility that the Roc proteins are acting redundantly to degrade Arm. To address this possibility, we are currently creating a fly stock that is mutant for both *Roc1b* and *Roc2*. Preliminary data suggests that these flies are also viable. We are also planning on creating *Roc1a* mutant clones in the *Roc1b* and the *Roc2* background, as well as the double mutant background.

Previous data by other labs have not shown that the Roc proteins can substitute for one another. Indeed, they have shown that the Rocs generally have preferred Cullin binding partners and don't stray from those (Reynolds et al., 2008). This made it very surprising that none of the three protein deficiencies by themselves could produce an accumulation of Arm. There remains the possibility that the original work done on *Roc1a* in flies might not have shown the whole picture. In *Noureddine 2002*, they showed that *Roc1a* is an essential gene, so mutant clones must be made in the fly in order to evaluate its affects. Further, clones lacking *Roc1a* have growth and proliferation defects. In order to have clones that were large enough to be able to evaluate the accumulation of Arm, the Duronio lab was forced to give the cells a very small amount of *Roc1a* to get them to live so that they could assess the phenotype. This method worked when they were testing a protein from the Hedgehog pathway, Cubitus interruptus (*Ci*). Clones that were mutant for *Ci* that were given a "shot" of the

protein still showed an accumulation of Ci. There is the possibility that the requirement of Roc1a for Arm degradation is much smaller. It could be that a very tiny amount is still enough to keep Arm levels low in these clones. David Roberts has generated data that suggests that Roc1a is responsible for degrading Arm in *Drosophila* S2 cultured cells (unpublished). This data has led us to attempt to recapitulate the Roc1a clones without the addition of Roc1a protein, to see if this is an artifact of tissue culture or a real possibility.

## **Materials and Methods**

### **Fly Stocks**

All experiments were done at 25°C. Mutations and Balancer chromosomes are described at FlyBase ([flybase.bio.indiana.edu](http://flybase.bio.indiana.edu)).

### **Generating Mutant Clones**

Clones were generated by FLP/FRT mediated mitotic recombination. Larvae were put at 37°C for 3 hours, 3 and 4 days after egg laying. After the heat shock, larvae were returned to 25°C for two days and then dissected.

### **Immunofluorescence**

We used a monoclonal mouse Anti-armadillo 7A1 raised against amino acids 67-123 (Developmental Studies Hybridoma Bank). Embryos were collected for two hours at 25°C, and then let age 5 hours (to stage 9). For larval collections 3<sup>rd</sup> instar larvae were dissected and the brains and wing discs were loosened from the cuticle to allow the antibody easier access. Embryos were fixed for 20 minutes in 10% formaldehyde in phosphate buffered saline (PBS). Larval tissues were fixed for 20 minutes in 4% formaldehyde in PBS. All collections were blocked for half an hour in 1% normal goat serum and 1% Triton X-100 in PBS

(PBT). Antibodies were diluted in PBT as follows:  $\alpha$ -arm 1:50, for larval brains and wing discs, 1:100 for embryos,  $\alpha$ -mouse 1:250 (Alexa by Molecular Probes). Primary antibodies were incubated at 4° overnight, secondary antibodies were incubated for three hours at 25°. Prior to mounting brains and wing discs were dissected completely from the cuticle. All samples were mounted in Aqua Poly/Mount (Polysciences). Fixed samples were imaged with a Pascal confocal microscope, using a Zeiss 40X NA 1.3 Plan-Neofluar oil immersion objective, and LSM software at 25°C. Adobe Photoshop CS2 was used to adjust input levels so the main range of signals spanned the entire output grayscale and to adjust brightness and contrast.

## **Western Blotting**

Embryos were collected for two hours and then aged 5 hours (stage 9, for both the Roc mutant western and the developmental western), or 21 hours (stage 17, developmental western). Brains and wing discs were dissected out from 3<sup>rd</sup> instar larvae. All samples were boiled for 5 minutes in 2x Laemmli buffer, run on an 8% acrylamide gel and transferred to a nitrocellulose membrane. The blot was incubated with  $\alpha$ -arm (1:75) and  $\alpha$ -tubulin (DM1A, 1:7500, Sigma) for one hour. Washes were done in Tris-Buffered Saline Tween-20 (TBST) at 4 x 15. For detection, the blot was incubated for one hour with horseradish peroxidase conjugated rabbit  $\alpha$ -mouse IgG secondary antibody (1:20000, Zymed), and then the ECL-Plus kit (GE Healthcare Amersham) was used.



## Northern Blotting

RNA was isolated with TRIzol (Sigma-Aldrich) from embryos (stage 9, stage 17) and brains and wing discs from 3<sup>rd</sup> instar larvae according to manufacturer's directions. A 1.5% agarose-formaldehyde gel was used to fractionate 3µg of each sample and then transferred to a nylon membrane. The prehybridization, hybridization, and posthybridization washes were done as described in Zinn et al 1983. Hybridization was done at 60°C. Probes for each transcript were made radiolabeling with either T7 (rp49, New England Biolabs) and T3 (arm, Promega). The membrane was simultaneously probed with riboprobes for *armadillo* and *rp49* as an internal control.

## Works Cited

Aberle, H., Bauer, A., Stappert, J., Kispert, A. and Kemler, R. (1997). B-catenin is a target for the ubiquitin-proteasome pathway. *The EMBO Journal* 16, 3797-3804.

Akong, K., Grevenkoed, E. E., Price, M. H., McCartney, B. M., Hayden, M. A., DeNofrio, J. C. and Peifer, M. (2002). *Drosophila* APC2 and APC1 Play Overlapping Roles in Wingless Signaling in the Embryo and Imaginal Discs. *Developmental Biology* 250, 91-100.

American Cancer Society "What Are the Key Statistics for Colorectal Cancer?" 2008. American Cancer Society. June 6, 2009 [http://www.cancer.org/docroot/cric/content/cric\\_2\\_4\\_1x\\_what\\_are\\_the\\_key\\_statistics\\_for\\_colon\\_and\\_rectum\\_cancer.asp](http://www.cancer.org/docroot/cric/content/cric_2_4_1x_what_are_the_key_statistics_for_colon_and_rectum_cancer.asp)

Brunner, E., Peter, O., Schweizer, L. and Basler, K. (1997). pangolin encodes a Lef-1 homolog that acts downstream of Armadillo to transduce the Wingless signal. *Nature* 385, 829-833.

Cadigan, K. M. (2008). Wnt-[beta]-catenin signaling. *Current Biology* 18, R943-R947.

Cavallo, R. A., Cox, R. T., Moline, M. M., Roose, J., Polevoy, G. A., Clevers, H., Peifer, M. and Bejsovec, A. (1998). *Drosophila* Tcf and Groucho interact to repress Wingless signalling activity. 395, 604-608.

Clevers, H. (2006). Wnt/[beta]-Catenin Signaling in Development and Disease. *Cell* 127, 469-480.

Donaldson, T. D., Nouredine, M. A., Reynolds, P. J., Bradford, W. and Duronio, R. J. (2004). Targeted Disruption of *Drosophila* Roc1b Reveals Functional Differences in the Roc Subunit of Cullin-dependent E3 Ubiquitin Ligases. *Mol. Biol. Cell* 15, 4892-4903.

Groden, J., Thliveris, A., Samowitz, W., Carlson, M., Gelbert, L., Albertsen, H., Joslyn, G., Stevens, J., Spirio, L., Robertson, M. et al. (1991). Identification and characterization of the familial adenomatous polyposis coli gene. *Cell* 66, 589-600.

Hamada, F., Tomoyasu, Y., Takatsu, Y., Nakamura, M., Nagai, S.-i., Suzuki, A., Fujita, F., Shibuya, H., Toyoshima, K., Ueno, N. et al. (1999). Negative Regulation of Wingless Signaling by D-Axin, a *Drosophila* Homolog of Axin 10.1126/science.283.5408.1739. *Science* 283, 1739-1742.

Hart, M., Concordet, J. P., Lassot, I., Albert, I., del los Santos, R., Durand, H., Perret, C., Rubinfeld, B., Margottin, F., Benarous, R. et al. (1999). The F-box protein [beta]-TrCP associates with phosphorylated [beta]-catenin and regulates its activity in the cell. *Current Biology* 9, 207-211.

Hayden, M. A., Akong, K. and Peifer, M. (2007). Novel roles for APC family members and Wntless/Wnt signaling during *Drosophila* brain development. *Developmental Biology* 305, 358-376.

Ho, M. S., Tsai, P.-I. and Chiang, C.-T. (2006). F-box proteins: the key to protein degradation. *Journal of Biomedical Science* Volume 13, 181-191.

Jiang, J. and Struhl, G. (1998). Regulation of the Hedgehog and Wntless signalling pathways by the F-box/WD40-repeat protein Slimb. 391, 493-496.

McCartney, B. M., Price, M. H., Webb, R. L., Hayden, M. A., Holot, L. M., Zhou, M., Bejsovec, A. and Peifer, M. (2006). Testing hypotheses for the functions of APC family proteins using null and truncation alleles in *Drosophila* 10.1242/dev.02398. *Development* 133, 2407-2418.

McMahon, A. P. and Moon, R. T. (1989). Ectopic expression of the proto-oncogene int-1 in *Xenopus* embryos leads to duplication of the embryonic axis. *Cell* 58, 1075-1084.

Munemitsu, S., Souza, B., Muller, O., Albert, I., Rubinfeld, B. and Polakis, P. (1994). The APC Gene Product Associates with Microtubules in Vivo and Promotes Their Assembly in Vitro. *Cancer Res* 54, 3676-3681.

Nathke, I. (2006). Cytoskeleton out of the cupboard: colon cancer and cytoskeletal changes induced by loss of APC. *Nat Rev Cancer* 6, 967-974.

Noureddine, M. A., Donaldson, T. D., Thacker, S. A. and Duronio, R. J. (2002). *Drosophila* Roc1a Encodes a RING-H2 Protein with a Unique Function in Processing the Hh Signal Transducer Ci by the SCF E3 Ubiquitin Ligase. *Developmental Cell* 2, 757-770.

Nusse, R. and Varmus, H. E. (1982). Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell* 31, 99-109.

Nusslein-Volhard, C. and Wieschaus, E. (1980). Mutations affecting segment number and polarity in *Drosophila*. 287, 795-801.

Ou, C. Y., Lin, Y. F., Chen, Y. J. and Chien, C. T. (2002). Distinct protein degradation mechanisms mediated by Cul1 and Cul3 controlling Ci stability in *Drosophila* eye development. *Genes Dev* 16, 2403-14.

Peifer, M. (1993). The product of the *Drosophila* segment polarity gene *armadillo* is part of a multi-protein complex resembling the vertebrate adherens junction. *J Cell Sci* 105, 993-1000.

Petroski, M. (2008). The ubiquitin system, disease, and drug discovery. *BMC Biochemistry* 9, S7.

Polakis, P. (2007). The many ways of Wnt in cancer. *Current Opinion in Genetics & Development* Genetic and cellular mechanisms of oncogenesis 17, 45-51.

Reynolds, P. J., Simms, J. R. and Duronio, R. J. (2008). Identifying Determinants of Cullin Binding Specificity Among the Three Functionally Different *Drosophila melanogaster* Roc Proteins via Domain Swapping. *PLoS ONE* 3, e2918.

Roberts, D. M., Slep, K. C. and Peifer, M. (2007). It takes more than two to tango: Dishevelled polymerization and Wnt signaling. *Nat Struct Mol Biol* 14, 463-465.

Riggelman B, Wieschaus E and P., S. (1989). Molecular analysis of the *armadillo* locus: uniformly distributed transcripts and a protein with novel internal repeats are associated with a *Drosophila* segment polarity gene. *Genes Dev.* 3, 96-113.

Rijsewijk, F., Schuermann, M., Wagenaar, E., Parren, P., Weigel, D. and Nusse, R. (1987). The *Drosophila* homology of the mouse mammary oncogene *int-1* is identical to the segment polarity gene *wingless*. *Cell* 50, 649-657.

Rubinfeld, B., Souza, B., Albert, I., Munemitsu, S. and Polakis, P. (1995). The APC Protein and E-cadherin Form Similar but Independent Complexes with alpha-Catenin, beta-Catenin, and Plakoglobin. *J. Biol. Chem.* 270, 5549-5555.

Sharma, R. P. and Chopra, V. L. (1976). Effect of the *wingless* (*wg1*) mutation on wing and haltere development in *Drosophila melanogaster*. *Developmental Biology* 48, 461-465.

van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A. et al. (1997). *Armadillo* Coactivates Transcription Driven by the Product of the *Drosophila* Segment Polarity Gene *dTCF*. *Cell* 88, 789-799.

van den Heuvel, M., Nusse, R., Johnston, P. and Lawrence, P. A. (1989). Distribution of the *wingless* gene product in *drosophila* embryos: A protein involved in cell-cell communication. *Cell* 59, 739-749.

Willems, A. R., Schwab, M. and Tyers, M. (2004). A hitchhiker's guide to the cullin ubiquitin ligases: SCF and its kin. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 1695, 133-170.

Yi Sun, M. T., Hangjun Duan, Manju Swaroop. (2001). SAG/ROC/Rbx/Hrt, a Zinc RING Finger Gene Family: Molecular Cloning, Biochemical Properties, and Biological Functions. *Antioxidants & Redox Signaling* 3, 635-650.

Zinn, K., Dimaio, D. and Maniatis, T. (1983). Identification of two distinct regulatory regions adjacent to the human [beta]-interferon gene. *Cell* 34, 865-879.